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Gulypyrones A and B and Phomentrioloxins B and C Produced by *Diaporthe gulyae*, a Potential Mycoherbicide for Saffron Thistle (*Carthamus lanatus*)

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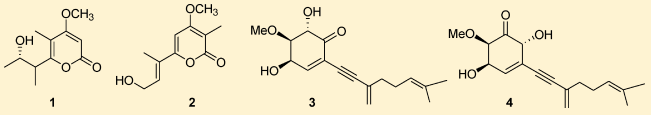
Supporting Information

ABSTRACT: A virulent strain of *Diaporthe gulyae*, isolated from stem cankers of sunflower and known to be pathogenic to saffron thistle, has been shown to produce both known and previously undescribed metabolites when grown in either static liquid culture or a bioreactor. Together with phomentrioloxin, a phytotoxic geranylcyclohexenetriol recently isolated from a strain of *Phomopsis* sp., two new phytotoxic trisubstituted α-pyrones, named gulypyrones A and B (**1** and **2**), and two new 1,*O*- and 2,*O*-dehydro derivatives of phomentrioloxin, named phomentrioloxins B and C (**3** and **4**), were isolated from the liquid culture filtrates of *D. gulyae*. These four metabolites were characterized as 6-[(2*S*)2-hydroxy-1-methylpropyl]-4-methoxy-5-methylpyran-2-one (**1**), 6-[(1*E*)-3-hydroxy-1-methylpropenyl]-4-methoxy-3-methylpyran-2-one (**2**), 4,6-dihydroxy-5-methoxy-2-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-2-enone (**3**), and 2,5-dihydroxy-6-methoxy-3-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-3-enone (**4**) using spectroscopic and chemical methods. The absolute configuration of the hydroxylated secondary carbon of the 2-hydroxy-1-methylpropyl side chain at C-6 of gulypyrone A was determined as *S* by applying a modified Mosher's method. Other well-known metabolites were also isolated including 3-nitropropionic, succinic, and *p*-hydroxy- and *p*-methylbenzoic acids, *p*-hydroxybenzaldehyde, and nectriapyrone. When assayed using a 5 mM concentration on punctured leaf disks of weedy and crop plants, apart from 3-nitropropionic acid (the main metabolite responsible for the strong phytotoxicity of the culture filtrate), phomentrioloxin B caused small, but clear, necrotic spots on a number of plant species, whereas gulypyrone A caused leaf necrosis on *Helianthus annuus* plantlets. All other compounds were weakly active or inactive.



Saffron thistle (*Carthamus lanatus* L. ssp. *lanatus*) is a widespread winter-growing annual weed of both pastures and crops throughout Australia and is considered the most economically important thistle species in New South Wales.[1,2] This weed has been declared noxious in all Australian States.[3] Poor results of mechanical[2] and chemical control have made this weed a suitable target for biological control.[2] In an effort to develop a mycoherbicide against this weed, pathogenic strains of *Phomopsis* sp. have been identified from diseased saffron thistle plants in Australia,[4] and their potential as mycoherbicides against the host has been evaluated.[5] Ash et al. (2010)[6] showed that this pathogen was distinct from other species of *Phomopsis* sp. commonly found on grain legume crops. More recently the teleomorph of this pathogen was identified as *Diaporthe gulyae* on the basis of morphology, DNA sequence analysis, and pathology studies,[7] and it has proved to be pathogenic to sunflower (*Helianthus annuus* L.), causing severe stem cankers.

Fungal metabolites have long been studied for their potential direct use as novel agrochemicals or as leads for new natural pesticides and to discover novel mechanisms of action.[8,9] The ability of *D. gulyae* to produce phytotoxic metabolites in culture was investigated, and a new phytotoxic geranylhydroquinone, named phomentrioloxin, was isolated as the main phytotoxic metabolite. It was characterized as (1*R*,2*R*,3*R*,4*R*)-3-methoxy-6-(7-methyl-3-methyleneoct-en-1-ynyl)-cyclohex-5-ene-1,2,4-triol using spectroscopic and chemical methods combined with X-ray analysis.[10]

Recently an SAR study was conducted using seven derivatives of the toxin that were tested for phytotoxic, antimicrobial, and zootoxic effects. Results from this study showed the hydroxy groups at C-2 and C-4, the cyclohexentriol, and to a lesser extent the unsaturations in the geranyl side chain are important for phytotoxicity.[11]

This article reports the chemical and biological characterization of gulypyrones A and B and phomentrioloxins B and C as potential natural safe herbicides from a new strain of *D.*

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gulyae. The influence of the fungal growth conditions and the use of a bioreactor on the metabolic profile are also discussed.

RESULTS AND DISCUSSION

The organic extract obtained from the culture filtrates of a new strain of *D. gulyae* grown in static culture proved to be highly phytotoxic. As reported in detail in the Experimental Section, its bioassay-guided purification revealed two new trisubstituted α-pyrones, named gulypyrones A and B (**1** and **2**, Figure 1, 0.3

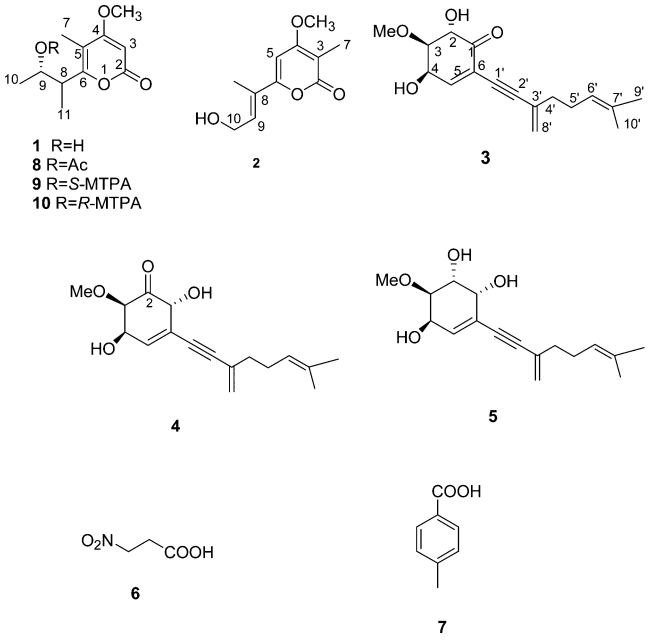

Figure 1. Structures of gulypyrone A (**1**); its 9-*O*-acetyl, 9-*O*-*S*-MTPA and 9-*O*-*R*-MTPA esters (**8**–**10**, respectively); gulypyrone B (**2**), phomentrioloxins B and C, and phomentrioloxin (**3**, **4**, and **5**, respectively); and 3-nitropropionic and 4-methylbenzoic acids (**6** and **7**, respectively) produced by *D. gulyae* grown in static culture.

and 0.13 mg/L, respectively), and two new analogues of phomentrioloxin (**5**, Figure 1, 0.85 mg/L), named phomentrioloxins B and C (**3** and **4**, Figure 1, 0.12 and 0.10 mg/L, respectively). The same culture filtrates also contained the well-known 3-nitropropionic and 4-methylbenzoic acids (**6** and **7**, Figure 1, 600 and 0.2 mg/L, respectively). 3-Nitropropionic acid was the main phytotoxic metabolite.

When the same fungus was grown in a bioreactor, the phytotoxic organic extract obtained from the culture filtrates yielded succinic acid (**11**, Figure 2, 193 mg/L) and 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and nectryapyrone (**12**–**14**, Figure 2, 2.72, 0.10, and 0.10 mg/L, respectively).

The identification of the metabolites 3-nitropropionic, succinic, and 4-hydroxy- and 4-methylbenzoic acids and 4-hydroxybenzaldehyde was confirmed by comparison of their TLC behavior and ^{1}H NMR spectra with those of the corresponding commercial samples. The structures of phomentrioloxin and nectriapyrone were confirmed by comparing their physical and spectroscopic data (OR, IR, UV, ^{1}H and ^{13}C NMR, ESIMS) with those reported in the literature for **5**[10] and **14**,[12] respectively.

Gulypyrone A (**1**) had a molecular formula of $C_{11}H_{16}O_4$ with four hydrogen deficiencies, as deduced by its HRESIMS. The IR spectrum showed the presence of hydroxy and α,β-unsaturated carbonyl groups[13] in agreement with the

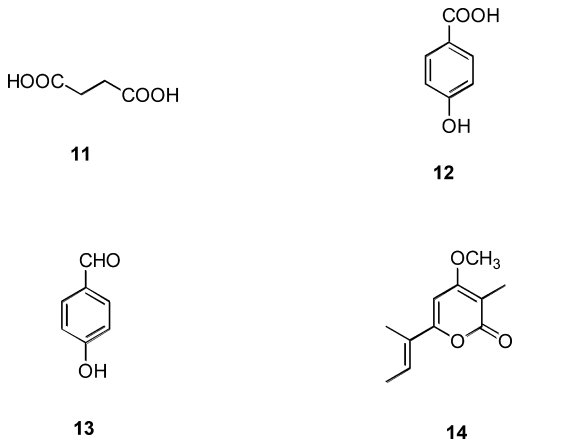

Figure 2. Structures of succinic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and nectriapyrone (**11**–**14**) produced by *D. gulyae* grown in a bioreactor.

absorption maximum at 288 recorded in the UV spectrum and typical of a substituted α-pyrone.[14] These results were in agreement with its ^{1}H and ^{13}C NMR spectra (Table 1). In particular, the ^{1}H NMR spectrum showed a singlet at δ 5.45 typical of an olefinic proton of a 4,5,6-trisubstituted α-pyrone ring.[15] The substituents appeared to be a methoxy, a vinyl methyl, and 1-methyl-2-hydroxypropyl residues. The methyl and the methoxy groups resonated as two singlets at δ 1.92 and 3.82, respectively. The protons of the 1-methyl-2-hydroxypropyl side chain, attributed on the basis of the couplings observed in the COSY spectrum,[16] appeared as a multiplet at δ 4.05 (J = 7.0 Hz), a quintet at δ 2.87 and two doublets (J = 6.2 and 7.0 Hz, respectively) at δ 1.27 and 1.19, respectively, and were attributed to H-9, H-8, Me-10, and Me-11, respectively.[15] The couplings observed in the HSQC spectrum[16] allowed the assignment of the corresponding carbons at δ 87.9, 56.1, 9.2 68.8, 42.7, 21.3, and 14.8 for C-3, OMe, C-7, C-9, C-8, C-10, and C-11, respectively.[17] The couplings observed in the HMBC spectrum[16] (Table 1) also allowed the assignment of the four quaternary carbons of the pyrone ring at δ 170.9, 165.1, 164.3 and 127.8 to C-4, C-6, C-2, and C-5, respectively. Thus, the chemical shifts of all the protons and the corresponding carbons of **1** were assigned as reported in Table 1.

The couplings observed in the same HMBC spectrum also allowed the positioning of the substituents on the α-pyrone ring. In particular, the $J^3_{C,H}$ and the $J^4_{C,H}$ couplings observed between OMe and C-4 and from Me-7 to C-4, C-5, and C-6, in agreement with the literature,[15−17] allowed their placement at C-4 and C-5, whereas the couplings between Me-11 and C-6 located the 2-hydroxy-1-methylpropyl side chain at C-6. These results were in agreement with the long-range coupling (<1 Hz) observed in the COSY spectrum between H-3 and both OMe and Me-7. On the basis of these results gulypyrone was formulated as 6-(2-hydroxy-1-methylpropyl)-4-methoxy-5-methylpyran-2-one.

The structure assigned to **1** was supported by the other couplings observed in the HMBC spectrum and from the HRESIMS and NOESY data. The HRESIMS showed the dimeric sodiated form $[2M + Na]^+$, the sodiated cluster $[M + Na]^+$, and the pseudomolecular ion $[M + H]^+$ at m/z 447, 235, and 213.1146, respectively. As expected the NOESY spectrum[16] showed correlations between OMe and H-3,

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Table 1. ^{1}H and ^{13}C NMR Data of Gulypyrones A and B (1 and 2)[a]

	1			2		
position	δC[b]	δH (J in Hz)	HMBC	δC[b]	δH (J in Hz)	HMBC
2	164.3 s			166.2 s		Me-11
3	87.9 d	5.45 (1H) s		104.3 s		H-5, Me-7
4	170.9 s		H-3, OMe, Me-7	165.6 s		OMe, H-5
5	127.8 s		H-3, Me-7	91.1 d	6.20 (1H) s	Me-11
6	165.1 s		Me-7, Me-11	159.2 s		Me-7
7	9.2 q	1.92 (3H) s		11.4 q	1.94 (3H) s	
8	42.7 d	2.87 (1H) quin (7.0)	Me-10, Me-11	128.1 s		H-5, H_2-10, Me-11
9	69.8 d	4.05 (1H) m	H-8, Me-10, Me-11	132.1 d	6.69 (1H) t (5.5)	H_2-10, Me-11
10	21.3 q	1.27 (3H) d (6.2)	H-8, Me-11	56.7 t	4.41 (1H) dd (1.0, 5.5)	
					4.39 (1H) dd (1.0, 5.5)	
11	14.8 q	1.19 (3H) d (7.0)	H-8, Me-10	14.3 q	1.93 (3H) s	
OMe	56.1 q	3.82 (3H) s		55.3 q	3.91 (3H) s	

[a]The chemical shifts are in δ values (ppm) from TMS. 2D ^{1}H,^{1}H (COSY) and ^{13}C,^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons. [b]Multiplicities were assigned by DEPT spectrum.

between H-9 and Me-10, and of H-8 with both Me-7 and Me-11.

The structure assigned to gulypyrone A was confirmed by preparing its 9-*O*-acetyl derivative (**8**). The IR spectrum of **8** showed the lack of the hydroxy group bands, while its ^{1}H NMR spectrum differed from that of **1** only by the downfield shift (Δδ 0.99) of H-9 resonating as a multiplet at δ 5.04 and the presence of the singlet of the acetyl group at δ 1.97. Its ESIMS showed the dimeric sodiated form $[2M + Na]^+$ and the potassium $[M + K]^+$ and sodium $[M + Na]^+$ clusters at *m*/*z* 531, 293, and 277, respectively. The APCI mass spectrum showed the pseudomolecular ion at *m*/*z* 255.

The absolute configuration of the secondary hydroxylated carbon (C-9) of the 2-hydroxy-1-methypropyl residue attached to C-6 was determined by applying a modified Mosher's method.[18] By reaction with *R*-(−)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) and *S*-(+)-MTPA chlorides, gulypyrone A (**1**) was converted into the corresponding diastereomeric *S*-MTPA and *R*-MTPA monoesters at C-9 (**9** and **10**, respectively), whose spectroscopic data were consistent with the structure assigned to **1**. Subtracting the proton chemical shifts (Table 2) of the 9-*O*-*R*-MTPA (**10**) from that of 9-*O*-*S*-MTPA (**9**) esters, the Δδ(**9**−**10**) values for all of the protons were determined as reported in Figure 3. The positive Δδ values were located on the right side, and those with negative values on the left side of the model A as reported by Othani et al. (1991).[18] This model allowed the assignment of the *S*

Table 2. ^{1}H NMR Data of (*S*)- and (*R*)-MTPA Esters of Gulypyrone A (9 and 10) in $CDCl_3$[a]

	9	10
position	$δ_H$ (J in Hz)	$δ_H$ (J in Hz)
3	5.314 s	5.414 (1H) s
7	1.697 (3H) s	1.767 (3H) s
8	3.105 (1H) quint (7.0)	3.119 (1H) quint (7.4)
9	5.272 (1H) quint (6.2)	5.266 (1H) quint (6.2)
10	1.441 (3H) d (7.0)	1.394 (3H) d (6.2)
11	1.187 (3H) d (6.2)	1.233 (3H) d (7.4)
OMe	3.755 (3H) s	3.119 (3H) s
OMe	3.512 (5H) s	3.373 (3H) s
Ph	7.41−7.31 (5H) m	7.33−7.30 (5H) m

[a]The chemical shifts are in δ values (ppm) from TMS.

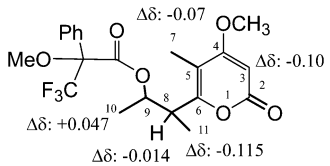

Figure 3. Structures of 9-*O*-*S*- and 9-*O*-*R*-MTPA esters of gulypyrone A (**9** and **10**), reporting the Δδ value obtained by comparison of each proton system.

configuration to C-9. **1** was named, according IUPAC nomenclature, as 6-((2*S*)-2-hydroxy-1-methylpropyl)-4-methoxy-5-methylpyran-2-one.

Gulypyrone B (**2**) had a molecular formula of $C_{11}H_{14}O_4$ with five hydrogen deficiencies, as deduced by its HRESIMS. Preliminary ^{1}H and ^{13}C NMR investigations, supported by the data from IR and UV spectra, showed that **2** is closely related to nectriapyrone (**14**, Figure 2), a phytotoxin recently isolated from culture filtrates of *Diaporthe angelicae* (anamorph *Phomopsis foeniculi*), a pathogen of fennel (*Foeniculum vulgare*) in Bulgaria.[12] The ^{1}H NMR spectrum (Table 1) showed a singlet at δ 6.20 typical of an olefinic proton of a 3,4,6-trisubstituted α-pyrone ring[15] and three further singlets at δ 3.91, 1.94, and 1.93, attributable to a methoxy and two vinyl methyl groups, respectively. Moreover ^{1}H NMR, COSY, and NOESY data proved that the two α-pyrones (**2** and **14**) differed only in the substitution of the 1-methylpropenyl side chain. The ^{1}H NMR spectrum of **2** showed the presence of a triplet (*J* = 5.5 Hz) at δ 6.69 assigned to the olefinic proton H-9, which in the COSY spectrum coupled with a hydroxymethylene group (H_2-10), that resonated as two doublets of doublets (*J* = 5.5 and 1.0 Hz) at δ 4.41 and 4.39, respectively. **2** differed from nectriapyrone by the presence of a hydroxy group at C-10 (Table 1), as also confirmed by the couplings observed in the HMBC spectrum.

The structure assigned to **2** was also supported by the NOESY data spectrum, in which a significant coupling was observed between H-5 with the methoxy group and Me-11. In the same spectrum a clear correlation was also observed between H_2-10 and Me-11, allowing the assignment of *E* stereochemistry to the trisubstituted double bond located between C-8 and C-9.

The structure assigned to **2** was supported by the data of the HRESIMS. The dimeric sodiated form, the sodium and

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potassium clusters, and the pseudomolecular ion were observed at m/z 443 $[2M + Na]^+$, 249 $[M + K]^+$, 233 $[M + Na]^+$, and 211.0985 $[M + H]^+$, respectively; the ESIMS spectrum also showed a significant fragmentation peak $[M + Na - CO_2]+$ at m/z 189 generated from the sodium cluster by loss of CO_2, a fragmentation mechanism typical of the α-pyrones.[15] **2** was named, according to IUPAC nomenclature, 6-[(1*E*)-3-hydroxy-1-methylpropenyl]-4-methoxy-3-methylpyran-2-one.

Phomentrioloxin B (**3**) had a molecular formula of $C_{17}H_{22}O_4$, as deduced by its HRESIMS, which is consistent with seven hydrogen deficiencies and therefore differed from phomentioloxin[10] by two hydrogen atoms. The IR spectrum of **3** differed from that of **5** essentially due to the presence of the band of a conjugated carbonyl group at 1734 cm^{-1}. The ^1H NMR spectrum (Table 3) of **3** differed from that of **5** by the lack of H-1 and HO-1 signals, while H-2 appeared as a double doublet (J = 10.0 and 2.0 Hz) at δ 4.57. Furthermore, the downfield shift ($\Delta\delta$ 0.98) of H-5 was observed as this proton resonated as a doublet (J = 6.0 Hz) at δ 7.14, the typical chemical shift of a proton of an α,β-unsaturated carbonyl group.[15] Considering these findings, the structure of a 1,*O*-dehydrophomentrioloxin might be suggested for phomentrioloxin B. The ^{13}C NMR data (Table 3) confirmed this hypothesis. The signals of the α,β-unsaturated carbonyl group appeared at δ 194.9 (C-1), 124.4 (C-6), and 146.4 (C-5) for the carbonyl group and the two carbons of the trisubstituted double bond, respectively.[17] Finally, the couplings observed in the COSY and HSQC spectra and the correlation observed in the NOESY spectrum between the Me-9′ and H-6′ allowed us to assign the chemical shifts to all the protons and their corresponding carbons (Table 2), and to phomentrioloxin B the structure 4,6-dihydroxy-5-methoxy-2-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-2-enone (**3**). This structure was supported by the long-range couplings observed in the HMBC spectrum (Table 3) and by the HRESIMS data. The latter, recorded in positive mode, showed, in addition to the dimeric sodiated form $[2M + Na]^+$, the potassium $[M + K]^+$ and sodium $[M + Na]^+$ clusters at m/z 603, 329, and 313.1427, respectively. The ESIMS spectrum was recorded in negative mode and showed the pseudomolecular ion $[M - H]^-$ at m/z 289.

Table 3. NMR Data of Phomentrioloxin B[a,b] and C[a] (3 and 4, Respectively)

	3			4
position	δC^c	δ_H (J in Hz)	HMBC	δ_H (J in Hz)
1	194.9 s		H-5, H-2	4.95 dd (11.0, 2.0)
2	73.7 d	4.57 (1H) dd (10.0, 2.0)	H-4, H-3	
3	81.0 d	3.42 (1H) dd (10.0, 4.0)	H-5, H-4, H-3, H-2	3.61 d (6.4)
4	63.9 d	4.65 (1H) ddd (6.0, 4.0, 1.9)	H-5	4.54 ddd (6.4, 6.0, 5.4)
5	146.4 d	7.14 (1H) d (6.0)	H-4	6.74 dd (6.0, 2.0)
6	124.4 s		H-4, H-5	
1′	81.5 s		H-5	
2′	95.7 s		H_2-8′	
3′	130.6 s		H_2-8′, H_2-4′	
4′	37.1 t	2.23 (2H) m	H_2-8′, H_2-5′	2.18 m
5′	26.7 t	2.23 (2H) m	H-6′, H_2-4′	2.18 m
6′	123.4 d	5.11 (1H) m	H_2-4′, H_2-5′	4.92 m
7′	123.1 s		Me-10′, Me-9′	
8′	123.4 t	5.46 (1H) d (1.4)	H_2-4′, H_2-5′	4.87 d (1.2)
		5.35 (1H) d (1.4)		4.81 d (1.2)
9′[d]	25.6 q	1.68 (3H) s	H-6′, Me-10′	1.74 s
10′[d]	17.7 q	1.63 (3H) s	Me-9′	1.64 s
OMe	59.2 q	3.65 (3H) s	H-3	3.65 s
HO-1				2.83 d (11.0)
HO-2		3.44 (1H) d (2.0)		
HO-4		2.86 (1H) d (1.9)		4.60 d (5.4)

[a]The chemical shifts are in δ values (ppm) from TMS. [b]2D ^1H,^1H (COSY) and ^{13}C,^1H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons. [c]Multiplicities were assigned by DEPT spectra. [d]These assignments could be reversed.

As depicted in Figure 1, the relative configuration of the chiral carbons of phomentriloxin B was deduced by the comparison of the coupling constants measured in its ^1H NMR spectrum with those of **5** and by the correlations observed in the NOESY spectrum between H-4 and both H-3 and H-5, as well as the lack of correlation between H-2 and H-3.[10]

Phomentrioloxin C (**4**) had the same molecular weight as phomentrioloxin B, as deduced by its ESI and APCIMS spectra, but its ^1H NMR spectrum (Table 3) differed from those of phomentrioloxin[10] and phomentrioloxin B. In particular, it differed from that of **5** essentially by the absence of the H-2 and HO-2 signals, while H-1 appeared as a doublet of doublets (J = 11.0 and 2.0 Hz) at δ 4.95. Compared to **3**, the spectrum did not show the same downfield shift observed for the signal of H-5 that resonated as a doublet of doublets (J = 6.0 and 2.0 Hz) at δ 6.74.

Considering these findings, the structure of a 2,*O*-didehydrophomentrioloxin might be suggested for phomentrioloxin C. This structure was supported by the ESI and APCIMS data. The ESIMS spectrum recorded in positive mode showed the dimeric sodiated form $[2M + Na]^+$ and the potassium $[M + K]^+$and sodium clusters $[M + Na]^+$ at m/z 603, 329, and 313, respectively. The ESIMS spectrum, recorded in negative mode, showed the pseudomolecular ion $[M - H]^-$ at m/z 289 and the ion it generated by loss of H_2O $[M - H - H_2O]^-$ at m/z 253. The APCI spectrum, recorded in positive modality, showed the pseudomolecular ion $[M + H]^+$ at m/z 291 and the ions originating from the latter by the loss of one or two H_2O molecules $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$ at m/z 273 and 255, respectively.

The relative configuration of phomentrioloxin C as depicted in Figure 1 was deduced by comparing the coupling constants measured in its ^1H NMR spectrum with those of **5**, and the trivial name phomentrioloxin B was given to 2,5-dihydroxy-6-methoxy-3-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-3-enone.

In the leaf disk bioassay, 3-nitropropionic acid proved to be quite active, being able to produce small but clear necroses on several weedy and crop plant species, in particular to *Papaver rhoes*, *Ecballium elaterium*, *Urtica dioica*, and *Hedysarum coronarium*. Smaller necroses were also visible on *Mercurialis annua*, *Lactuca serriola*, *Ailanthus altissima*, and *Inula viscosa*. Moreover, considering that the compound is present at higher concentrations, it is probably the principal compound responsible for the high phytotoxicity of the culture filtrates and the organic extract. Tested at the same concentration, phomentrioloxin B proved to have a weaker toxicity, causing necrosis on *P. rhoeas* and *U. dioica*, and even smaller necrosis on

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M. annua, *L. serriola*, *A. altissima*, *Picris echioides*, *I. viscosa*, *H. coronarium*, *H. annuus*, and *Aster* sp. Assayed at 5 mM by stem immersion on *H. annuus* plantlets, gulypyrone A caused the rapid appearance of clear and very expanded necrosis on leaves; interestingly, the lack of symptoms on the stems suggests that the compound can be easily translocated through the vascular system, accumulating in the leaf tissues. Even weaker activity was observed in the case of 4-methylbenzoic acid, which caused small necrosis only to *M. annua*, *U. dioica*, *Solanum nigrum*, and *Aster* sp. All the other compounds were very weakly or not at all toxic. Considering the high toxicity of the culture filtrate, an additive or synergistic activity of all the weakly active metabolites could be hypothesized.

None of the compounds showed significant antibacterial or antifungal activity.

Gulypyrones A and B belong to a group of naturally occurring compounds that are broadly distributed, being produced by plants, animals, marine organisms, and microbes and having interesting biological properties.[19−21] These include metabolites containing the pyran-2-one moiety produced by fungi belonging to several genera and showing antibiotic, antifungal, cytotoxic, neurotoxic, and phytotoxic activities.[22] Other bioactive fungal α-pyrones have been isolated, including viridepyronone produced by *Trichoderma viride*,[23] having antifungal activity against *Sclerotium rolfsii*, the causal agent of crown and stem rot of artichoke. 3-Nitropropionic acid was previously isolated as a phytotoxin from other fungi pathogenic to weeds.[24] *p*-Hydroxybenzaldehyde is a known phytotoxic metabolite of pathogenic fungi of important crops and forest plants. This metabolite was also isolated together with known cytochalasins from the liquid and solid cultures of two strains of *Phoma exigua* var. *exigua*, a pathogenic fungus that has been proposed for the biocontrol of *Cirsium arvense* and *Sonchus arvensis*.[25] *p*-Hydroxybenzoic acid, identified as a phytotoxin produced by *Rhizoctonia oryzae*, induced brown spots typical of disease on plant tissue.[26] *p*-Methylbenzoic acid was isolated from cultures of the fungus *Cladosporium cladosporioides* isolated from a marine sponge.[27]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in a $CHCl_3$ solution on a Jasco P-1010 digital polarimeter; IR spectra were recorded as a glassy film on a PerkinElmer Spectrum One FT-IR spectrometer, and UV spectra were recorded in MeCN solution on a PerkinElmer Lambda 25 UV/vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 400, and at 125 and 100 MHz, respectively, in $CDCl_3$ on Bruker spectrometers. The same solvent was used as an internal standard. Carbon multiplicities were determined by DEPT spectra.[16] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[16] were performed using Bruker microprograms. HRESIMS spectra were recorded on Thermo LTQ Velos, and ESI and APCIMS spectra were recorded on Agilent Technologies 6120 Quadrupole LC/MS instruments. Analytical and preparative TLC were performed on silica gel plates (Merck, Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) or on reversed-phase (Whatman, KC18 F_{254}, 0.20 mm) plates; the spots were visualized by exposure to UV light and/or iodine vapors and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, and/or by spraying with 0.5% ninhydrin in Me_2CO and/or bromocresol green 0.04% in EtOH followed by heating at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.063−0.200 mm).

Fungal Strain. The strain of *Diaporthe gulyae* used in this study was isolated from symptomatic saffron thistle (*C. lanatus*) plants in Australia[7] and stored in the fungal collection of the Istituto di Scienze delle Produzioni Alimentari, CNR, Italy, with the code ITEM 54025. The DNA sequence of this strain of *D. gulyae* was reported previously by Ash et al. 2011.[7] Its GenBank accession numbers are ITS, JF431299; TEF-1α, JN645803. The fungus was routinely grown on PDA plates at 25 °C as starting material for the production of culture filtrates.

Extraction and Purification of Phytotoxins Produced in Static Culture. The strain of *D. gulyae* used in this study was grown on a minimal defined liquid medium named M1-D[28] as previously reported.[10] The culture filtrates (27.7 L, pH 4.5), having high phytotoxic activity on leaves, were lyophilized, dissolved in distilled water (1/10 of its original volume), and then extracted by EtOAc (4 × 3 L) previously acidified to pH 2.5 with HCOOH. The organic extracts were combined, dehydrated with anhydrous Na_2SO_4, and evaporated under reduced pressure, yielding a brown solid (20.3 g), showing high phytotoxic activity. This extract was dissolved in EtOAc and then washed with a saturated solution of $NaHCO_3$ to remove 3-nitropropionic acid identified as the main metabolite (**6**, 18.4 g, 692 mg/L, Figure 1). The organic phase was dehydrated with anhydrous Na_2SO_4 and evaporated under reduced pressure, affording a brown, oily residue (1.07 g), which showed phytotoxic activity. This oil was purified by CC developed with $CHCl_3$−*i*-PrOH (9:1), yielding eight groups of homogeneous fractions. The residue of the third fraction (51.8 mg) was further purified by TLC, developed with $CHCl_3$−*i*-PrOH−acetone (93:5:2), giving four fractions. The residue of the second fraction obtained as a yellow oil was characterized, as below reported, as phomentrioloxin C (**4**, 2.7 mg, 0.10 mg/L, R_f 0.49, Figure 1). The residue of the third fraction (5.6 mg) was purified by TLC, developed with $CHCl_3$−*i*-PrOH (95:5), giving a white, amorphous solid identified as 4-methylbenzoic acid (**7**, 5.4 mg, 0.20 mg/L, R_f 0.54, Figure 1). The residue of the fourth fraction (86.3 mg) of the first chromatographic column was purified by TLC developed with $CHCl_3$−*i*-PrOH−acetone (93:5:2), giving four homogeneous fractions. The residue of the second fraction (10.3 mg) was further purified by TLC on reversed-phase developed with EtOH−H_2O (7:3). One metabolite was obtained as a yellow oil, characterized as phomentrioloxin B (**3**, 3.3 mg, 0.12 mg/L, R_f 0.57, Figure 1) as reported below. The fifth fraction of the first chromatographic column (120.9 mg) was further purified by TLC developed with $CHCl_3$−*i*-PrOH (9:1). Two metabolites were obtained as homogeneous solids, one identified as gulypyrone A (**1** 7.0 mg, 0.3 mg/L, R_f 0.46, Figure 1), as below reported, and the other one identified as phomentrioloxin (**5**, 23.0 mg, 0.85 mg/L, R_f 0.32, Figure 1).[10] The sixth fraction (89.6 mg) of the first chromatographic column was purified by TLC eluted with $CHCl_3$−*i*-PrOH (95:5), giving five homogeneous fractions. The residue of the third fraction (7.3 mg) was further purified by TLC on reversed-phase developed with EtOH−H_2O (7:3). One metabolite was obtained as a white solid characterized as gulypyrone B (**2**, 3.5 mg, 0.13 mg/L, R_f 0.46) as reported below.

Gulypyrone A (1), 6-((2*S*)-2-hydroxy-1-methylpropyl)-4-methoxy-5-methylpyran-2-one: amorphous solid; $[\alpha]^{25}_D$ −46.5 (*c* 0.2); IR ν_{max} 3407, 1694, 1635, 1560, 1453, 1405, 1246 cm^{-1}; UV λ_{max} nm (log ε) 288 (3.94); ^{1}H and ^{13}C NMR see Table 1; HRESIMS *m*/*z* 447 [2M + Na]$^+$, 235 [M + Na]$^+$, 213.1146 [calcd for $C_{11}H_{17}O_4$ 213.1137, M + H]$^+$.

9-*O*-Acetyl Derivative of 1 (8). Gulypyrone A (**1**, 0.5 mg) dissolved in pyridine (10 μL) was acetylated with acetic anhydride (10 μL) at room temperature for 1 h. The reaction was stopped by addition of MeOH, and the azeotrope, obtained by the addition of benzene, was evaporated by an N_2 stream. The oily residue (2.0 mg) was purified by preparative TLC developed with $CHCl_3$−*i*-PrOH (97:3), to give 9-*O*-acetylgulypyrone as a homogeneous compound (**8**, R_f 0.62, 0.8 mg). Derivative **8**: IR ν_{max} 1731, 1643, 1563, 1541, 1245 cm^{-1}; UV λ_{max} nm (log ε) 283 (2.04); ^{1}H NMR, δ 5.44 (1H, s, H-3), 5.04 (1H, m, H-9), 3.81 (3H, s, OMe), 3.13 (1H, quint, *J* = 7.0 Hz, H-8), 1.97 (3H, s, MeCO), 1.94 (3H, s, Me-7), 1.30 (3H, *J* = 6.2 Hz, Me-10), 1.22 (3H, d, *J* = 7.2 Hz, Me-11); ESIMS (+) *m*/*z* 531 [2M + Na]$^+$, 293 [M + K]$^+$, 277 [M + Na]$^+$; APCIMS, *m*/*z* 255 [M + H]$^+$.

(*S*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate Ester of Gulypyrone A (9). Gulypyrone A (**1**, 0.5 mg) was converted into the corresponding MTPA ester (**9**) by reaction with (*R*)-(−)-MTPA-

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Cl (20 μL) in dry pyridine (20 μL). The usual reaction workup yielded **9** as a homogeneous solid (0.9 mg): IR ν_{max} 1744, 1713, 1648, 1566, 1454, 1248 cm^{-1}; UV λ_{max} nm (log ε) 286 (2.68); ^1H NMR see Table 2; ESIMS (+) *m/z* 879 $[2M + Na]^+$, 467 $[M + K]^+$, 451 $[M + Na]^+$; APCIMS *m/z* 429 $[M + H]^+$.

(*R*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate Ester of Gulypyrone A (10). Gulypyrone A (**1**, 0.5 mg) was converted into the (*R*)-MTPA ester of **1** (**10**) by reaction of (*S*)-(+)-MTPA-Cl. The reaction was carried out under the same conditions used for preparing **9** from **1**. **10**, obtained as a homogeneous solid (0.9 mg): IR 1745, 1711, 1648, 1566, 1454, 1245; UV λ_{max} nm (log ε) 286 (2.71); ^1H NMR see Table 2; ESIMS (+) *m/z* 879 $[2\times M + Na]^+$, 467 $[M + K]^+$, 451 $[M + Na]^+$; APCIMS *m/z* 429 $[M + H]^+$.

Gulypyrone B (2), 6-((1*E*)-3-hydroxy-1-methylpropenyl)-4-methoxy-3-methylpyran-2-one: amorphous solid; IR ν_{max} 3239, 1694, 1625, 1553, 1421, 1370, 1262 cm^{-1}; UV λ_{max} nm (log ε) 228 (4.55), 323 (4.12); ^1H and ^{13}C NMR see Table 1; HRESIMS (+) *m/z* 443 $[2M + Na]^+$, 249 $[M + K]^+$, 233 $[M + Na]^+$, 211.0985 [calcd for $C_{11}H_{15}O_4$ 211.0970, $M + H]^+$, 189 $[M + Na - CO_2]^+$; ESIMS (−) *m/z* 209 $[M - H]^-$.

Phomentrioloxin B (3), 4,6-dihydroxy-5-methoxy-2-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-2-enone: yellow oil; $[\alpha]^{25}_D$ +1.9 (*c* 0.2); IR ν_{max} 3862, 2207, 1734, 1695, 1458 cm^{-1}; UV λ_{max} nm (log ε) 223 (2.84), 278 (2.69), 399 (2.19); ^1H and ^{13}C NMR see Table 3; HRESIMS (+) *m/z* 603 $[2M + Na]^+$, 329 $[M + K]^+$, 313.1427 [calcd for $C_{17}H_{22}NaO_4$ 313.1416, $M + Na]^+$; ESIMS (−) *m/z* 289 $[M - H]^-$.

Phomentrioloxin C (4), 2,5-dihydroxy-6-methoxy-3-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-3-enone: yellow oil; IR ν_{max} 3831, 2214, 1752, 1673, 1424 cm^{-1}; UV λ_{max} nm (log ε) 231 (2.73) 276 (2.62), 397 (2.05); ^1H NMR see Table 3; ESIMS (+) *m/z* 603 $[2M + Na]^+$, 329 $[M + K]^+$, 313 $[M + Na]^+$; ESIMS (−) *m/z* 289 $[M - H]^-$, *m/z* 253 $[M - H - H_2O]^-$; APCIMS (+) *m/z* 291$[M + H]^+$, 273 $[M + H - H_2O]^+$, 255 $[M + H - 2H_2O]^+$.

Extraction and Purification of Phytotoxins Produced in a Bioreactor. The same strain of *D. gulyae* was also grown in a bioreactor (BIOSTAT C 30, Sartorius Stedim Biotech). Two 1 L Erlenmeyer flasks, each containing 500 mL of PDB (potato-dextrose broth), were inoculated with the actively growing mycelium obtained from six plates of PDA and kept for 2 days in shaking conditions (100 rpm) on a orbital rotary shaker. The whole culture containing the mycelium was then fragmented in sterile conditions with a homogenizer for 2 min. The suspension was injected into the bioreactor (filled with 30 L of *in situ* sterilized M1D medium) with the aid of a peristaltic pump and maintained for 2 weeks at a temperature of 26 °C, 80 rpm, and 1 L/min air flux. The culture (30 L, pH 4.5) was then collected, filtered first through gauze and then through filter papers to remove mycelium, and lyophilized. The lyophilized culture filtrates was extracted in the same conditions as previously reported, yielding a brown solid (7.3 g), which was highly phytotoxic. The extract was dissolved in EtOAc and then washed with a saturated solution of $NaHCO_3$ to remove acid compounds using the same procedure as previous reported, giving the mixture of acid compounds as a brown solid (6.4 g) and the neutral and/or basic compounds as a yellow oil (340.0 mg). The acid mixture was washed with small aliquots of EtOAc to separate as white solids of succinic acid (5.8 g, 190 mg/L) and 4-hydroxybenzoic acid (**11** and **12**, 82.5 mg, 2.75 mg/L, R_f 0.32, Figure 2). The fraction containing neutral and/or basic compounds has been purified by CC developed with $CHCl_3$−*i*-PrOH (9:1), yielding eight groups of homogeneous fractions. The residue of the second fraction (109.3 mg) was further purified by CC, developed with $CHCl_3$−*i*-PrOH−acetone (94:3:3), giving five homogeneous fractions. The residue of the second fraction obtained as a homogeneous white solid was identified as nectriapyrone (**14**, 7.2 mg, 0.10 mg/L, R_f = 0.45, Figure 2). The residue of the third fraction (15 mg) of the first CC was purified by TLC, developed with *n*-hexane−-EtOAC (50:50), giving four fractions. The residue of the fourth fraction obtained as a white solid was characterized as 4-hydroxybenzaldehyde (**13**, 2.1 mg, 0.10 mg/L, R_f 0.49).

Biological Activities. In order to evaluate the presence of specific toxins, culture filtrates, organic extracts, and samples were assayed on punctured detached leaves of *Helianthus annuus*, one of the hosts of the pathogen, as described elsewhere. This bioassay was then used to guide all of the purification steps. The pure metabolites (except phomentrioloxin C, which was not available in sufficient amounts) were assayed at 5 mM on 15 plant species, both cultivated and weedy. Leaf disks (diameter 9 mm) were obtained by using a cork borer, placed on wet filter paper in polycarbonate boxes, and punctured in the middle by using a syringe needle. A droplet (15 μL) of the toxin solution was applied on each punctured disk. Boxes were kept at 25 °C under continuous light for 3 days. Four replications were prepared for each metabolite and for each plant species. Control disks were prepared with the same procedure but were added with water droplets. Effects were evaluated as necrosis appearance after 3 days by using a visual empiric scale from 0 (= no necrosis) to 4 (necrosis 8−10 mm). For the antibacterial activity pure compounds were tested on *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, whereas the antifungal activity was tested on *Geotrichum candidum* as described elsewhere.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR and HRESIMS spectra are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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